PAPER NO. 9

COMPARISON OF ANALYSIS AND QUANTIFICATION OF CELL DEATH IN VIVO AND IN VITRO

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INTRODUCTION

Exogenous chemicals may interact incidentally with many of the cell's subcellular processes--storage and transmission of genetic information, membrane transport and permeability, energy production, synthesis of macromolecules, etc.--as the involved cell attempts to dispose of the foreign agent by metabolically altering it and, at the same time strives to maintain its functional integrity and viability. Cellular toxic responses occur when the affected cells are no longer able to maintain functional and structural equilibrium as a result of the chemically induced perturbation of one or more of the cell's essential metabolic processes (Grisham and Smith, 1984; Smuckler and James, 1984). Cellular toxicity is a broad and complex subject; the manifestations of chemical toxicity at the level of the individual cell comprise a variety of abnormalities in structure and function of affected cells that range in severity from cell death, expressed by sudden lysis of cells, to mild and transient alterations in synthetic or metabolic functions (Grisham and Smith, 1984). Included among the more frequently studied manifestations of cytotoxicity by chemicals that damage DNA are mutation and neoplastic transformation. Cell death is far more important to the overall health of the organism than is either somatic mutation or neoplastic transformation, for example, occurring much more frequently than does either of these more commonly studied cytotoxic responses. Chemicals can kill cells by a variety of mechanisms in addition to those that involve perturbation of the accurate transmission of genetic information; many chemicals kill cells even though they are not mutagenic or carcinogenic. Furthermore, even chemicals that are strong mutagens/carcinogens kill cells much more often than they mutate or transform them.

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SESSION III

IN VITRO APPROACHES TO IN VIVO TOXICITY: GENERAL PRINCIPLES

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We have recently examined the quantitative relationships among cell death (including reproductive failure), mutation, and neoplastic transformation in mouse 10T1/2 cell populations cultured in vitro (in which all three of these cytotoxic endpoints can be accurately quantified), in response to exposure to monofunctional alkylating chemicals that damage DNA and other cellular molecules (Grisham et al., 1980; Grisham and Smith, 1984; Smith et al., unpublished observations, 1984). At doses of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) that reduced the relative colony forming efficiency of 10T1/2 cells by less than 60%, 3,000 to 6,000 cells were killed or reproductively sterilized for each cell that was mutated at the Na+/K+ATPase locus (Oua^S Oua^r) or that was transformed to produce a type III focus. At higher doses of MNNG, cell death predominated even more strikingly, with an average of about 20,000 cells killed or reproductively sterilized for each cell mutated or neoplastically transformed at chemical doses that reduced relative colony forming efficiency by at least 75%. At higher concentrations of MNNG, all cells were killed and none were mutated or transformed. Our work indicates that MNNG kills, sterilizes, mutates, and transforms 10T1/2 cells by a mechanism or mechanisms that depend on similar lesions in DNA (Smith and Grisham, 1983; Smith et al., unpublished observations, 1984). The predominance of cell killing over mutation and neoplastic transformation was even more extreme when the toxic chemical was methyl methane sulfonate (MMS), a monofunctional alkylating agent that kills 10T1/2 cells mainly by damaging their cell membranes although it produces a spectrum of DNA damage identical to that produced by MNNG (Smith and Grisham, 1983). As toxic responses to MMS-induced damage to 10T1/2 cells, mutation and neoplastic transformation were extremely rare events at any level of cell killing studied. The differences in toxic responses to MNNG and MMS appear to relate to their different alkylation mechanisms; MMS more heavily alkylates molecules in cell membranes than does MNNG (Smith and Grisham, 1983). Without going into the details of alkylation mechanisms, these observations show the relatively marked predominance of cell death as a toxic response to this class of chemical mutagens/carcinogens. Our recent studies indicate that this relationship between cell killing and mutation or transformation also extends to other chemical classes of mutagens/carcinogens (Smith et al., unpublished observations, 1984).

From the standpoint of the organism, induced cell death is of obvious importance. Massive death of cells in essential organs and tissues is not compatible with continued life of the organism. Also, cell death triggers a variety of reparative processes, including proliferation of the remaining viable cells in the damaged population and of various stromal cells. The

reparative processes that are triggered by induced death of cells are themselves mechanistically involved in the production of slowly developing chronic lesions, including fibrosis and cancer, which may impair the health and result in the death of the organism.

Even though cell death is a major toxic consequence of chemical injury, this cytotoxic response is not as widely applied as an endpoint to assess the toxicity of chemicals and to evaluate their mechanisms of toxicity as the relative frequency and importance of cell death would appear to merit. This relative neglect of cell death as quantitative endpoint of cytotoxicity assays appears to have resulted mainly from the technical difficulty to sensitively identify and accurately quantify dead cells, especially in vivo. The aim of this presentation is to discuss some of the major problems and possibilities encountered in evaluating cell death in tissues and organs in vivo and in cultured cell populations in vitro, by comparing and contrasting the identification and enumeration of dead cells in the two settings.

GENERAL ASPECTS OF CELL DEATH

Cell death is a constant feature of healthy tissues in the living organism and of populations of unperturbed cells in culture. All mammalian somatic cells appear to have a finite life span that is genetically programmed (Hayflick, 1977). Following the consummation of this program the cell dies a natural, physiological, or scheduled death. Little is known about the mechanism of scheduled death of cells, but this event occurs constantly in all cell populations that proliferate, either in vivo or in vitro. Death of cells other than those that have reached the end of their genetic program, is termed unscheduled, induced, accidental, or pathological. Death of cells resulting from exposure to toxic chemicals is unscheduled. When evaluating unscheduled death of cells induced by chemicals, one must be aware of the potentially confounding effect of scheduled death.

Until relatively recently, the only means of identifying dead cells was morphologic. The classic morphologic marker of the dead cell is the observed destruction of the cell nucleus (pyknosis, karyorrhexis, karyolysis), which is coupled with swelling and dissolution of the cell or with its progressive shrinkage and apparent desiccation. These changes are termed necrosis; necrosis is not identical to cell death, but represents the morphologic alterations that occur in the dead cell as its structure is progressively degraded by the action of catabolic

enzymes whose activities have been able to survive the death of the cell (Majno et al., 1960). Some investigators distinguish between dead cells that undergo swelling necrosis and those that undergo shrinkage necrosis (also termed apoptosis), under the assumption that different mechanisms are involved (Wylie et al., 1980). At present there is little convincing evidence of major mechanistic differences between these two morphologic responses other than the rather obvious conclusion that swelling necrosis is associated with changes in membrane permeability. Programmed cell death is said to more often be followed by shrinkage necrosis than is unscheduled cell death (Wylie et al., 1980), but this distinction is not absolute (shrinkage necrosis commonly involves hepatocytes that have been killed by either viral or chemical toxins [Zimmerman, 1978]). The apparent association between swelling necrosis and chemical exposure may result from the fact that many chemicals are able to damage the cell membrane, increase its permeability to ions, and allow water to accumulate in the cell (Zimmerman, 1978).

Classic morphologic criteria of cell death (necrosis/apoptosis) have great precision, but little sensitivity, for identifying dead cells. Although cells that exemplify these morphologic features are certainly dead, many dead cells may not demonstrate these morphologic changes. Necrosis and apoptosis are alterations in cell structure that follow cell death, and the speed with which these degenerative changes develop may vary widely depending on the local milieu in which the affected cell is located (Majno et al., 1960). Furthermore, necrotic cells are ultimately destroyed completely and may leave little evidence of their former presence. As a consequence, the use of morphologic criteria, either in vivo or in vitro, will inevitably underestimate the magnitude of dead cells, failing to indicate those cells that are dead but have not been degraded sufficiently to be detected morphologically and those cells that died concurrently but are already totally destroyed. Not only does the use of morphologic criteria of necrosis prevent the accurate quantitation of the magnitude of cell death, these criteria also make it impossible to determine the moment of cell death (the "point of no return" when cell injury becomes irreversible). This fact severely handicaps the mechanistic analysis of the cellular processes involved in the induction of cell death by chemicals and makes it difficult to distinguish those events that cause the cell to die from those that only follow its death.

During the past thirty years, many attempts have been made to improve the sensitivity for detecting cell injury and for identifying and quantifying dead cells. Associated with the various damages to cellular processes that are induced by

chemicals and lead to cell injury and death are a variety of functional aberrations, including failure to maintain ATP levels with associated structural changes in mitochondria, breakdown of the cellular organelles for protein synthesis, accumulation of partly completed metabolic products, such as lipids or proteins, and alterations in membrane permeability with subsequent accumulation by the cell of extracellular ions and water (Grisham and Smith, 1984). Even though these functional manifestations of cell injury always accompany chemically induced cell death, many of them represent reversible injuries that have little sensitivity or specificity for predicting impending or indicating actual cell death. Of the functional changes that appear to be most specifically associated with cell death, alteration in the permeability properties of the cell membrane appears to be a good, but not absolute, predictor (Smith and Grisham, 1983; Grisham and Smith, 1984). The ability to proliferate, either continuously or on demand, is an essential property of most cells in vivo or in vitro and this functional property is sensitive to the injurious action of many chemicals, especially those that damage DNA (Smith and Grisham, 1983). The ability of a tissue to maintain a critical population of functioning cells is vital, and its interdiction is a sensitive indication of cytotoxicity. Population size is determined by the fraction of cells that leave the population by programmed or unscheduled cell death and by the rate of cell birth. For continuously proliferating populations of cells, reproductive sterilization is the functional equivalent of cell death, although reproductively sterilized cells may continue to carry out some synthetic and metabolic functions.

CELL DEATH IN VIVO

It is necessary to evaluate chemically-induced cell death in vivo in order to determine the identity and location of target cells and tissues. At present there is no acceptable substitute for the intact animal for determining the combined effects of distribution and organ-specific metabolism of the test chemical on the location and relative number of dead cells (Grisham and Smith, 1984). Although primary cultures of parenchymal cells from various organs may be used to study the metabolism of protoxic chemicals and to evaluate the effect of metabolites on some cellular functions, in vitro cell systems cannot reproduce the pharmacodynamic complexity of the animal.

Dead cells are identified in tissues and organs in vivo by the morphological criteria of necrosis and apoptosis, previously discussed. Morphological evaluation of tissues from animals

requires histological or cytological examination, which necessitates that relevant tissues be sampled by biopsy or autopsy and the specimens appropriately fixed and stained. Morphologic criteria of nuclear disintegration coupled with cell swelling or shrinkage are necessary for firm identification of dead cells in vivo. A helpful histologic feature for the identification of dead cells is the presence of injured cells together with an infiltration of acute inflammatory cells, which are stimulated to migrate into the area by signals released from dead cells. Macroscopic areas of necrotic cells may be identified with the unaided eye by the characteristic changes in color and consistency that groups of dead cells undergo during their catabolic destruction in vivo; these grossly visible changes are collectively known to the pathologist as coagulative necrosis. Quantitation of the area (and volume) occupied by dead cells in tissues and organs can be determined by applying standard morphometric techniques (Baak and Oort, 1983), provided that the sample of tissue evaluated is representative of the entire organ. It is difficult or impossible to convert the morphometric measurements to total numbers of dead cells because of possible differential shrinkage of dead and living cells during tissue fixation and dehydration. Even when this problem can be satisfactorily met, the volume of the whole organ and the number of cells/unit volume must be known. Usually morphometry can provide only semiquantitative estimates of the magnitude of cell death. The presence and fraction of cells with permeable membranes can be evaluated by dye exclusion assays modified for use in vivo (Balinsky et al., 1984).

Alterations in the levels of cellular metabolic enzymes in blood plasma (or serum) may allow the detection of cell injury and necrosis in the animal, and occasionally the identification of the particular organ or tissue in which the necrosis is located (Wilkinson, 1970). Enzymes of cellular metabolism are present in cells in high concentration, but their concentration in blood is very low in the healthy animal. When cells die or are sufficiently injured that their membranes become permeable to proteins, the soluble metabolic enzymes leak from the cells into the extracellular fluid and are ultimately carried into the blood, from which they are later cleared by excretion or catabolism (Wilkinson, 1970). In those uncommon instances in which an enzyme or isoenzyme is unique or nearly so for a particular cell or organ, the presence of this enzyme or isoenzyme in the blood can be used to identify target cell and organ. Enzymes that are more or less organ-specific and may be used to identify the site of tissue necrosis include sorbitol dehydrogenase and alcohol dehydrogenase (hepatocytes), acetylcholinesterase (erythrocytes and neurons), alkaline phosphatase (osteoblasts),

and acid phosphatase (prostatic epithelium). Analysis of certain isoenzymes in blood may improve the localization of the organ or tissue that contains necrotic cells (Wilkinson, 1970). Isoenzymes of lactate dehydrogenase have been most extensively studied and may be useful in identifying the site of necrosis in one of several possible cellular sites (Wilkinson, 1970). Creatine kinase isoenzymes are especially useful in identifying myocardial necrosis and may be used to roughly estimate the relative size of the area of myocardial necrosis (Chapman and Silverman, 1982). Unfortunately for the widespread application of analysis of cellular enzymes in blood as a means to identify target tissues, most cellular metabolic enzymes are not specific to different types of cells, but are relatively ubiquitous in their tissue distribution (Wilkinson, 1970). Although the measurement of cellular metabolic enzymes and isoenzymes in blood may offer considerable assistance in clinical diagnostic situations, this technique does not provide a useful means to accurately quantify the magnitude of cellular necrosis in routine toxicity assays.

Accurate quantification of cell death in organs and tissues in vivo remains to be attained in practice. Use of morphologic markers of cell death (necrosis/apoptosis) leads to the serious underestimation of the magnitude of cell death. Cytostatic toxic responses (reproductive failure) are impossible to directly detect in vivo by morphologic means because the affected cells are viable. Cellular reproductive failure may be detected in vivo only by secondary effects in proliferating tissues, as indicated by hypoplasia of stem cells leading to atrophy of the affected tissue. Such tissues as bone marrow, gastrointestinal epithelium, seminiferous tubules, and others may show dramatic secondary atrophy as a result of death or reproductive sterilization of the stem cell precursors of the differentiated functioning cells. In some limited situations it is possible to evaluate the proliferation kinetics of cell populations in tissues by autoradiographically detecting radiolabeled precursors of DNA in sequential studies (Aherne et al., 1977). These techniques are very demanding, often requiring several animals for each time point and requiring rather massive labor. It is not feasible to use these methods as part of a routine assay for chemical toxicity, but only as a part of a focused experimental study. As an experimental procedure, it is possible to quantitatively dissociate cells of certain tissues with an enzymatic solution and subject the isolated cells to flow cytometric analysis, as discussed in the next section.

CELL DEATH IN VITRO

Cultured cell populations allow the investigator to comprehensively account for the fate of all cells in populations exposed to toxic chemicals. The cytotoxic responses that affect population growth include cell death, reproductive sterilization, and altered rates of cycle transit (Grisham and Smith, 1984). Populations of cultured cells grow exponentially continuously if they are provided with sufficient nutriment and not allowed to become confluent. Population growth results from a dynamic balance reflecting the rates of cell birth and death. The rate of cell birth depends on the fraction of the population that is proliferating (cycling) and the length of the cell cycle (or frequency of cycling) of the cells in the proliferating (growth) fraction. All of these properties of proliferating populations can be quantified by applying classic methods of population analysis (Aherne et al., 1977) or analogous mechanized methods using flow cytometry (FCM) (Gray, 1983). The classic methods, while accurate, are tedious and time consuming. FCM techniques allow the examination of literally thousands of cells from sampled populations, and the data may be readily analyzed by available computer programs. FCM methods may be validated by the concurrent application of classic methods to analyze the kinetics of cell population growth.

Using FCM or classic methods for analyzing the role of individual cells in population growth, combined with techniques to evaluate cell permeability and to assay clonogenicity, a balance sheet for the entire population may be constructed. One evaluates membrane permeability in individual cells, manually (Grisham and Smith, 1984) or by FCM (Gray, 1983), determines clonogenicity (RCFE) (Grisham and Smith, 1984), and analyzes the population growth rate. Increased membrane permeability is a good indicator of cell death by chemicals or other agents that damage cell membranes (Philips, 1973), but it may not reflect dead cells killed by other mechanisms or reproductively sterilized cells and it does not always correlate with the RCFE (Roper and Drewinko, 1976; Grisham and Smith, 1983). RCFE provides a precise measure of the fraction of rapidly cycling cells in the population that remain after death and reproductive sterilization of affected cells. The only remaining data needed to construct the balance sheet is the estimation of the fraction of cells that show slowed cycling and the determination of the average cycle time for this group. A rough estimate of the magnitude of slowed cycling versus dead or reproductively sterilized cells can be determined by inspection of the population growth curves (Grisham and Smith, 1984). Growth curves that show a dose-dependent variation in slopes suggest a dose-dependent slowing of cell cycle

transit, whereas those that show parallel, displaced slopes (with the extent of displacement reflecting dose) indicate that cell killing and/or reproductive sterilization have predominated. Of course, curves for the growth of chemically exposed populations may show combinations of these changes, indicating complex cytotoxic responses (Grisham and Smith, 1984). The growth fraction, the average cycle time, and range of variation in cycle times can be determined rapidly by routine FCM methods (Gray, 1983). Furthermore, FCM methods also allow the rapid determination of the point in the cycle at which reproductively sterilized cells are blocked (Gray, 1983).

Although cultured cells may be used to kinetically analyze the toxic responses of proliferating cells to chemical exposures, most propagable cell lines do not metabolize protoxic chemicals to the range of toxic metabolites that are formed in vivo (Grisham and Smith, 1984). Continuously, propagable cell lines are, therefore, virtually limited to the analysis of toxicity of directly active chemicals. Primary cultures of target cells retain the ability to metabolize protoxic chemicals to the toxic metabolites encountered in vivo, but cannot be used to analyze the full range of cellular toxic responses since cells in most short-term primary cultures do not proliferate (Grisham and Smith, 1984). It may be possible to develop co-cultures of primary isolates of metabolically competent cells from target organs and continuously propagable cell lines in which cell death can be accurately quantified. Previous use of such co-culture systems for evaluating the toxic consequences of protoxic chemicals that require metabolic activation has been limited to the evaluation of genotoxic responses, especially mutation (Langenbach et al., 1978). Techniques that will allow the quantitation of cytotoxic responses in indicator cells without interference by metabolizing cells remain to be developed. Other strategies for accomplishing this goal include the use of lines derived from tumor cells that can both activate protoxic chemicals and be used to quantitate toxic responses (Loquet and Weibel, 1982), and the genetic engineering of metabolically competent indicator cells by transferring active genes for chemical metabolism into continuously culturable cell lines.

DISCUSSION

Cell death and related cytotoxic responses are frequent cellular reactions to toxic chemicals. Cell culture systems allow more accurate quantitation of cell death and related reactions in response to chemicals than do animals. However, mammalian cell culture systems do not yet provide a solution for

many requirements of toxicity testing. Cell culture systems are unable to reflect distribution and organ-specific metabolic activation of protoxic chemicals that are typical of different animals species. Thus, cell culture systems do not replace the need for animals in toxicity studies, but when used in conjunction with limited studies in animals cell culture assays can improve the sensitivity of detection of chemical toxicity, and provide more detailed understanding of cellular mechanisms of toxic responses. Before cell cultures can be used to replace animals in assaying the toxicity of chemicals, culture systems must be developed that maintain in vivo levels of metabolic function and culture methods must be standardized to optimize specificity, sensitivity, and reproducibility of response. It is also necessary that the accuracy of measuring toxic responses in culture systems to predict toxicity in animals be fully validated by studies employing direct animal-culture comparisons with chemicals of various classes before culture systems can be accepted as surrogates for animals.

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